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PRINCIPAL INVESTIGATOR:

Fei Du

CONTRACTING ORGANIZATION:
California, University of, San Diego
La Jolla, CA 92093

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Fei Du

E-Mail: fedu@ucsd.edu

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14. ABSTRACT

The RasGAP domain only accounts for a small fraction of the NF1 sequence and many evidence suggest that NF1 is involved in other cellular functions besides inactivating Ras. Our NF1 epidermal knockout mice provide us a unique model system to understand the relevance of the Ras pathway and of other pathways in relation to the NF1 gene. To identify Ras-independent pathways of NF1, we analyzed the NF1 protein complex formed in the mouse keratinocytes by immunoprecipitation followed by Mass Spectrometry analysis. We identified 22 novel NF1 interacting proteins which can be grouped to 7 categories. Unfortunately we didn't identify any protein with obvious function in differentiation and inflammation, but we found some proteins involved in neuron development, Ras signaling and skin diseases. After analyzing four different stages of differentiation, we conclude that NF1 involves in keratinocyte migration and the initiation of differentiation in epidermis. Furthermore, knockout NF1 in epidermis results in increased inflammatory responses in old mice indicating that the loss of NF1 contributes to inflammatory response but a second factor such as age is required to fulfill this process. Moreover, the skin disease we observed in those mice is psoriasis.

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Neurofibromin (NF1), Ras, epidermis, immunoprecipitation, differentiation, inflammation

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Introduction

To date, there is no cure for Nf1 and treatments are aimed at controlling the symptoms. Surgery can help some NF1 bone malformations and remove painful or disfiguring tumors; however, there is a chance that the tumors may grow back and in greater numbers (*www.ninds.nih.org*). Therefore, studies on the functions of NF1 and its signaling pathways are critical to uncover new drug targets for Nf1. Clinical trials on the drugs to lower the abnormal increased levels of Ras, such as lovastatin, simvastatin and farnesyltransferase inhibitors Tipifarnib are ongoing (*www.ctf.org*) (*1, 2*). However trials showed that simvastatin failed to improve the cognitive function in children with Nf1 (*3*) and Tipifarnib therapy showed little efficacy in the treatment of NF1-associated plexiform neurofibroma (*2*). Since the RasGAP domain only accounts for a small fraction of the NF1 sequence, finding other genes that affect the function of the NF1 gene is crucial to identify new drug target to cure Nf1. In the NF1 epidermal knockout mice we generated, the loss of NF1 in the basal layer of the epidermis didn't lead to the hyperplasia of the proliferating layer as we expected. Instead, we saw the expansion of the spinous layer which is composed of differentiated keratinocytes. Furthermore, we detected hyper-activation of Ras in both heterozygous and homozygous mice with NF1 mutation but the epidermal phenotypes were only observed in the full knockouts. Thus, our NF1 epidermal knockout mice provide us a unique model system to understand the relevance of the Ras pathway and of other pathways in relation to the NF1 gene. The ultimate goal of this research program is to find other proteins regulated by NF1 to provide further insights into the pathogenesis of Nf1 and uncover potential molecular targets for controlling clinical manifestations in Nf1 patients.

Body

To identify Ras-independent pathways involved in NF1 function, we proposed to analyze the NF1 protein complex formed in the mouse keratinocytes by immunoprecipitation followed by Mass Spectrometry analysis and focus on the Ras-independent signaling pathways involved in the differentiation and inflammatory response because of the epidermal phenotypes we observed.

Task 1: Perform mass spectrometry analysis of NF1 complex in mouse keratinocytes (months 1-12).

1a. *Proposed work: Perform immunoprecipitation assay with NF1 antibody together with a crosslinker and precipitate the NF1 complex from wild-type keratinocytes (months 1-4). Keratinocytes from wild-type mice (established cell lines in the lab) will be used to test the efficiency of the antibodies in terms of western blotting and immunoprecipitation.*

To perform immunoprecipitation assay we need a good NF1 antibody. A good NF1 antibody is the one giving clear bands around 220kd by western blotting only in the cell lysates from epidermis of wild-type and NF1 heterozygous mice but not the one from NF1 conditional knockout mice. Also with immunofluorescence staining, the antibodies should only stain the epidermis of wild-type and NF1 heterozygous but not homozygous with NF1 mutant. As we reported last year, we have tested 6 commercial NF1 antibodies, including NB300-155 (Novus Biologicals), 200150 (Abbiotec), sc-20982, sc-67, sc-68 and sc-20016 (Santa Cruz Biotechnology) and 22 ascites fluid from different hybridomas generated by Abmart using western blotting and immunofluorescence staining. The #14 ascites fluid showed potential but weak positive results with both methods. To verify this result, we did western blotting of NF1 with mouse brain samples where NF1 predominantly expresses (*4, 5*). Unfortunately, #14 ascites fluid didn't give a recognizable band. However, sc67 from Santa Cruz Biotechnology detected a decent band at the right size. To further confirm the specificity of the sc67 antibody, we performed immunoflurorescence staining of skin samples from NF1 epidermal knockout mice and their wild-type littermates. As shown in figure 1A, the murine skin consists of two basic components, an epithelial compartment comprised of stratified epithelial cells (keratinocytes) and an underlying mesenchymal compartment, separated by an elaborate extracellular matrix, the basement membrane. Epidermal homeostasis is maintained by the constant proliferation of stem cells residing in the basal layer. When cells withdraw from the cell cycle, they may initiate a program of terminal differentiation to form the biochemically distinct layers of the epidermis (figure 1A) (*6*). Using sc67 antibody for immunofluorescence

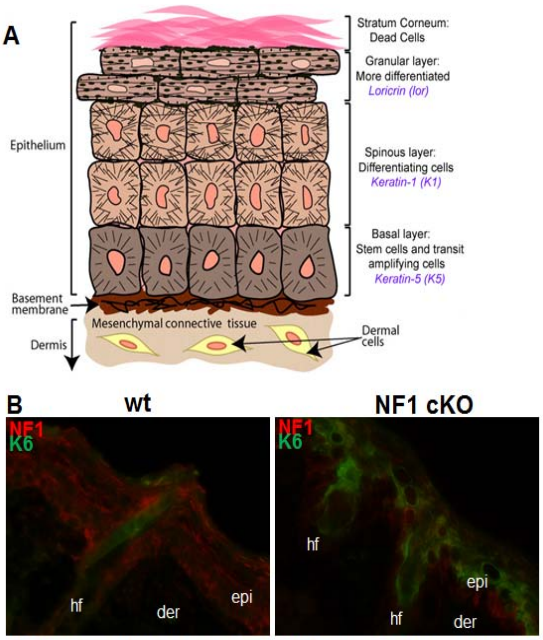

Figure 1: Expression of NF1 in murine skin. (A) Murine skin structure. The skin is separated into two compart-ments, the epidermis and dermis by the basement membrane. The epidermis is stratified epithelia of proliferating and differentiating cells that can be divided into distinct layers by different markers (purple). Adapted from Alonso and Fuchs, 2003 (6). (B) Immunofluorescence staining of keratin 6 and NF1 detected by sc67 antibody in skin samples from postnatal 21 day wild-type (wt) and NF1 epidermal knockout mice (NF1 cKO). Epi: epidermis, der: dermis, hf: hair follicle.

staining, we detected distinct basal layer staining of NF1 (figure 1B) which is consistent with previous report (*7*). However, the basal layer staining was also strong in NF1 epidermal knockout mice (figure 1B) suggesting that the basal cell staining we observed might not be a real NF1 staining, but rather a non-specific readout. Interestingly, we also observed clear spinous and granular layer staining of NF1 in wild-type skin which was lost in NF1 with homozygous mutant (figure 1B). Similar spinous and granular layer expression of NF1 in human skin was also mentioned in an academic dissertation but the result was never published maybe due to lack of quality control (*8*) . Here, we stained skin with keratin 6 to show the region where NF1 gene is lost and epidermal homeostasis is disrupted. Keratin 6 is a marker for skin abnormality. In wild-type skin, keratin 6 only expresses in hair follicles and when the skin function is disturbed, keratin 6 expresses in epidermis (*9, 10*). As shown in figure 1B, we observed epidermal expression of keratin 6 in NF1 conditional knockout, where we can still detect robust basal layer staining but not spinous and granular layer staining of NF1. Consistently, in the epidermal region of NF1 conditional knockout where keratin 6 is not expressed, we can still see staining in spinous and granular layer (data not shown). It suggests that NF1 is highly expressed in differentiated mouse keratinocytes and sc67 antibody is a good NF1 antibody, which might be suitable for immunoprecipitation.

1b. *Proposed work: Clone full length NF1 with a tag into keratinocytes expression vector and transfect the DNA into mouse keratinocytes (month 3-4). If the antibodies we test are not efficient to do the pulldown assay we will perform this subtask.*

We cloned the full length NF1 with a T7 tag at the 5' into a mKTs expression vector under the K14 promoter. Unfortunately the expression level was too low to detect by western blotting using T7 antibody. Since we did find a good NF1 antibody, we didn't pursue further to increase the exogenous expression of NF1.

1c. *Proposed work: Perform immunoprecipitation assay and precipitate the protein complex (months 5-6). If subtask 1a succeeds, we will extract keratinocytes from new-born mice including NF1 epidermal knockout mice, NF1 epidermal heterozygous mice and their wild-type littermates and use those cells to do immunoprecipitation assay. If we performed subtask1b, keratinocytes transfected with tagged-NF1 will be used for the immunoprecipitation instead.*

When we used mouse epidermis lysates on western blotting with sc67 antibody, we didn't see clear NF1 band, but rather with high background (data not shown). However we detected distinct NF1 band with mKT lysate. Since NF1 is highly expressed in the spinous and granular layer where differentiated mKTs reside, we examined the expression of NF1 in mKTs after differentiation induced by high calcium and high confluency. We saw significant increase of the expression of NF1 after differentiation (figure 2A) which further proved that the spinous and granular layer staining in epidermis is a real staining whereas the basal staining was non-specific binding. This observation also explains that NF1 epidermal knockout mice have strong phenotype in spinous layer but not basal layer. Thus, we used differentiated mKTs for our pull-down assay. By using sc67 conjugated agarose (sc67-AC), we pulled down many specific proteins compared to the rabbit IgG conjugated to agarose as a control including a protein around 280Kd, presumably the NF1 band as shown by silver staining of the immunoprecipitated samples (figure 2B). This band was also detected positively with sc67 antibody by western blotting (figure 2C). Normally crosslinkers enhance the protein-protein interaction, thus we tested immunoprecipitation in the presence of a reversible chemical crosslinker, Dithiobis-Succinimidylpropionate (DSP), but we even didn't detect NF1 in the immunoprecipited product (data not shown). This might due to the formation a big complex in the presence of crosslinkers which blocked the access to isotope of the NF1 by the antibody. After immunoprecipitation, we

eluted the proteins from agarose beads by 4% SDS/0.1M Glycine (PH 2.5). After incubation with 100mM DTT and neutralizing the elution with PH8.0 ThisHCl, the proteins was precipitated the proteins with methanol/chloroform (*11*). The precipitated proteins were analyzed by Zhouxin Shen in Steven Briggs lab in UCSD.

1d. *Proposed work: Mass spectrometry analysis of the NF1 complex (months 6-12). The purified proteins will be sent to the Steven Briggs lab in UCSD and the mass spectrometry analysis will be performed by Zhouxin Shen. The data will be analyzed against murine protein database and potential NF1 interacting proteins will be identified. Information including the gene name, molecular weight, spectra count, total chromatogram intensity and total number of identified distinct peptides will be collected.*

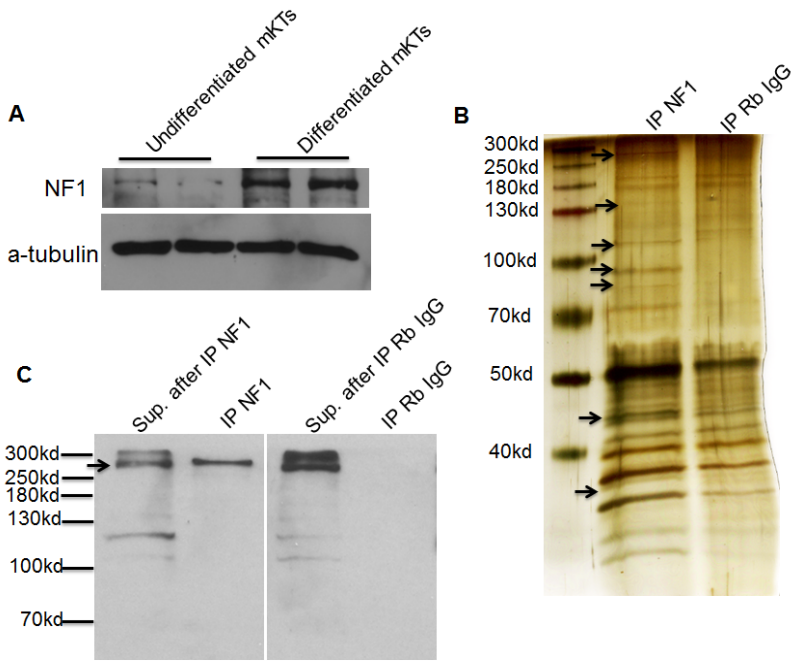

Figure 2: Immunoprecipitation of NF1 complex. (A) Increased expression of NF1 after differentiation of mKTs by high calcium and high confluency. (B) Silver staining of immunoprecipitation product after pulldown with NF1 anti-body and rabbit IgG control in differentiated mKTs. Arrows indicate the proteins pull-downed by NF1 antibody only. (C) Western blotting of NF1 in supernatant and immunoprecipitation product after pulldown with NF1 antibody and rabbit IgG control in differentiated mKTs. Arrow indicates NF1 band.

The samples were washed with acetone, resuspended in Tris buffer, 8 M urea, pH 8.6, reduced with 100 mM TCEP, and alkylated with 55 mM iodoacetamide. Trypsin digest was done in the presence of 1 mM $CaCl_2$ for tryptic specificity. Peptide mixtures were loaded onto a triphasic LC/LC column with the following steps of 500 mM ammonium acetate bumps: 25, 35, 50, 80, and 100%. Tandem mass spectra were analyzed using DTA Select and the mouse sequence database with the following filtering parameters for cross correlation scores 1.8 (+1), 2.8 (+2), and 3.5 (+3) (*12*). Identities of specific bands were confirmed by sequence analysis. Proteins that are uniquely identified in the after immunoprecipitation with NF1 antibody were listed in table 1.

Task 2: Study NF1 signaling pathway involved in the differentiation (months 13-24). We will selectively study the proteins involved in epidermal differentiation using the criteria described below.

2a. *Proposed work: Narrow down the proteins list against the proteins involved in epidermal differentiation (month 13). Immunoprecipitation and mass spectrometry analysis always reveal many potential interacting proteins but we will focus on the epidermal differentiation by screening the list of the microarray data from Blumenberg lab.*

As shown in table 1, we could roughly divide the proteins identified in the mass spectrometry analysis to 7 groups, as Ras related proteins, neuron proteins, cytoskeleton associated proteins, skin specific proteins, nuclear proteins and transcription factors, few metabolism proteins and proteins involved in cell surface receptor expression and EGFR trafficking. It is not surprising to uncover proteins involved in Ras signaling pathway since NF1 is a known Ras-GAP. The discovery of 5 proteins involved in neuron growth and development might shed lights on the tumorigenesis of neurofibroma and neurofibromatosis type 1 related cognition defect. Furthermore, it has been reported that NF1 co-localizes with actin filament structures in many types of cells and tissues including mKTs and skin (*7, 8, 13*). We did detect some of the cytoskeleton proteins as potential NF1 binding proteins (table 1). The identification of Corneo-desmosin precursor (CDSN) explains the localization of NF1 in spinous layer of epidermis and some of the phenotypes we observed which we will discuss in details below. Moreover, as reported, NF1 has strong perinuclear and nuclear staining in some cell lines (*8, 14, 15*) and we observed robust perinuclear localization of NF1 in mKTs as well (data not shown). This observation justifies the discovery of many nuclear proteins and transcription factors in the NF1 complex (table 1). The finding of proteins regulating cell surface receptor expression and trafficking, such as EGFR, might elucidate the belief that EGFR plays an important role in NF1 peripheral nerve tumorigenesis (*16-18*). Although the purpose of this study was to identify differentiation and inflammation related proteins in NF1 signaling pathway, we didn't find any protein with obvious roles in differentiation nor inflammation.

Symbol	Proteins
Ras related proteins	
G3bp2	Isoform B of Ras GTPase-activating protein-binding protein 2
G3bp2	Isoform A of Ras GTPase-activating protein-binding protein 2
G3bp1	Ras GTPase-activating protein-binding protein 1
Neuron proteins	
Dpysl3	Dihydropyrimidinase-related protein 3
Dpysl2	Dihydropyrimidinase-related protein 2
Sip1	Survival of motor neuron protein-interacting protein 1
Ss18l1	Synovial sarcoma translocation gene on chromosome 18-like 1
Caprin1	Cytoplasmic activation/proliferation-associated protein 1
Cytoskeleton associated proteins	
Dpysl3	Dihydropyrimidinase-related protein 3
Tpm4	Tropomyosin alpha-4 chain
Skin specific protein	
Cdsn	Corneodesmosin precursor
Nuclear proteins and transcription factors	
Nono	Isoform 1 of Non-POU domain-containing octamer-binding protein
Nup214	nucleoporin 214
Hnrpu	heterogeneous nuclear ribonucleoprotein U
Ddi2	Rsc1a1 DDI1 homolog 2
Eif4b	Eukaryotic translation initiation factor 4B
Pspc1	Paraspeckle protein 1
Tdrd3	Tdrd3 protein
Thoc4	Isoform 1 of THO complex subunit 4
Ss18l1	Synovial sarcoma translocation gene on chromosome 18-like 1
Caprin1	Cytoplasmic activation/proliferation-associated protein 1
Metabolism proteins	
Paics	Multifunctional protein ADE2
Hsd17b4	Peroxisomal multifunctional enzyme type 2
Miscellanies	
Gipc1	PDZ domain-containing protein GIPC1
Atxn2	Isoform 2 of Ataxin-2

Table 1: Mass spectrometry analysis of the NF1 complex. The NF1 containing complex was immunoprecipitated and analyzed by mass spectrometry to determine the NF1-interacting proteins. Rabbit IgG were used as a control. Proteins that were uniquely identified by anti-NF1 antibody were listed in the table. We divided the proteins to 7 groups some of which were overlapped.

2b. *Proposed work: In case we still have too many proteins to study, we will further narrow down the list by yeast-2-hybrid screening (months 14-22). If we will identify too many proteins involved in epidermal differentiation, we will use the RasGAP domain of NF1 as a bait and*

screen against the keratinocytes cDNA library. The genes identified from the yeast-2-hybrid screening will be ruled out from our list.

This part of the work was not done because we didn't discover any protein involving in cell differentiation.

2c. *Proposed work: Characterize the protein of interest (months 14-24). By co-immunoprecipitation assay we will confirm that the POI can pull-down NF1. We will determine the expression pattern of this protein in the epidermis by immunofluorescence or immunochemistry staining both in wild-type and NF1 epidermal knockout mice. We will compare the RNA and protein level of the POI in the epidermis of NF1 knockout mice and wild-type mice by RT-PCR and western blotting. We will read literatures and find out if there is any disease or mouse model related to the gene which encodes the protein and if there is any similarity between the phenotypes reported and the ones in Nf1.*

We didn't detect any NF1 interacting protein with noticeable function in differentiation, so we did some preliminary study on skin differentiation. When keratinocytes initiate differentiation, there are four steps occur, as cell cycle arrest, detach from underlying basement membrane, outward migration and initiation of terminal differentiation (*19*). If any of the steps goes wrong, the balance between proliferation and differentiation is disrupted which results in either hyperproliferation or precocious differentiation. We decided to dissect if NF1 is involved in any specific stage.

We did immunofluorescence staining on ki67, a cellular marker for proliferation, which presents during all active phases of the cell cycle (G_1, S, G_2, and mitosis), but is absent from resting cells (G_0) (*20*). There is no difference of the proliferation in the basal layer of NF1 epidermal knockout compared to their wild-type littermates or NF1 heterozygous mice (data not shown). This is consistent with the observation of the normal basal layer in the conditional knockout mice and further proves that NF1 plays some Ras-independent role in epidermis.

When the basement membrane is disrupted, keratinocytes tend to detach from the underlying basement membrane and leads to early differentiation (*21*). We performed immunofluorescence staining of β4-integrin, one of the key components of the basement membrane and found the basement membrane is intact in the NF1 conditional knockout mice (data not shown).

NF1 has been reported to regulate cell migration. For example, Schwann cells derived from Nf1-null mice have enhanced chemokinetic and chemotactic migration in comparison to wild-type controls (*22*). Using the small interfering RNA (siRNA) technique, it is demonstrated that NF1 dynamically regulates actin cytoskeletal reorganization, followed by enhanced cell motility and gross cell aggregation in Matrigel matrix in HeLa and HT1080 cells (*23*). Furthermore, homozygous Nf1 mutant (Nf1–/–) Schwann cells secrete Kit ligand (KitL), which stimulates mast cell migration (*24*). If NF1 regulates keratinocytes migration as in some other cells, downregulation the expression of NF1 would lead to increased cell motility which promotes the stratification and results in pro-differentiation as we saw in the NF1 epidermal knockout mice. We tested this hypothesis by extracting skin explants from NF1 epidermal knockout mice and their wild-type littermates and measure the distance of keratinocytes migrated from the explants. We examined the skin explant migration on 21 days old mice and found that the keratinocytes migrate more slowly in NF1 epidermal knockout mice compare to wild-type mice (figure 3A). There is one report that loss of NF1 in *Dictyostelium* leads to impaired cell migration and chemotaxis (*25*). But the slower migration ability in NF1 knockout mice cannot explain the precocious differentiation phenotypes we observed. In the meanwhile, we investigated the expression of some genes involved in migration in epidermis including MMP3, MMP9, uPAR, integrinα3, integrinβ6, and TGFβ1 and found no significant change of the expression those genes, except MMP3 (figure 3B). The expression of MMP3 is greatly reduced in NF1 epidermal

knockout mice compared to wild-type which might be the cause of slower explant migration from NF1 epidermal knockout mice (figure 3) (*26-31*).

Loss of NF1 in epidermis causes the expansion of spinous layer, which composed of differentiated cells. To determine if NF1 regulates terminal differentiation or just the initiation of the differentiation, we examined the terminal differentiation process of NF1 by checking the formation of corneocytes.

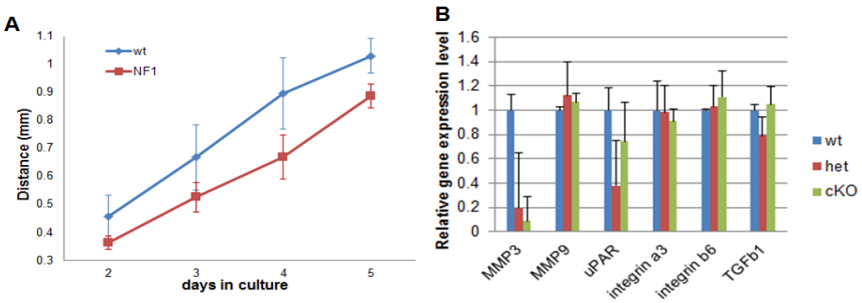

Figure 3: NF1 negatively regulates migration in mKTs. (A) Distance between the explant edge and the most distal cells of the outgrowth was measured over time. (B) Quantitative real-time PCR of gene expression involved in cell migration of newborn epidermis in wild-type (wt), NF1 with heterozygous (het) or homozygous mutation (cKO) mice. Gene expression was normalized to transcripts of β-actin. Gene expression of wt was set at 1.

Corneocytes are terminal differentiated, dead, and flattened cells which form the stratum corneum, the outmost layer of epidermis and functioning as a protective barrier (*32*). We extracted corneocytes from the epidermis of NF1 epidermal knockout mice and their wild type littermates and found the corneocytes were similar both in shape and number (data not shown). We also measured the transepidermal water loss and confirmed the intactness of the skin barrier (data not shown). Altogether, our data suggest that that NF1 regulates the initiation of differentiation in the epidermis but the mechanism is still unknown.

Task 3: Determine the role of NF1 in the inflammatory response (months 1-24). We will selectively study the proteins involved in inflammation from the mass spectrometry data.

3a. *Proposed work: We will go through literatures and pick the proteins involved in the inflammation identified by the mass spectrometry analysis (month 13).*

As shown in table 1, we didn't identify any proteins clearly involved in inflammatory response.

3b. *Proposed work: Characterize the protein of interest (months 14-24). By co-immunoprecipitation assay we will confirm that the POI can pull-down NF1. We will determine the expression pattern of this protein in the epidermis by immunofluorescence or immunochemistry staining both in wild-type and NF1 epidermal knockout mice. We will compare the RNA and protein level of the POI in the epidermis of NF1 knockout mice and wild-type mice by RT-PCR and western blotting. We will find out if there is any disease or mouse model related to the gene which encodes the protein and if there is any similarity between the phenotypes reported and the ones in Nf1.*

Not done since we didn't identify any proteins involved in inflammatory response.

3c. *Proposed work: Determine the inflammatory profile in the NF1 knockout mice (months 1-12). Perform immunofluorescence staining on pNFκB, CD3 (T-cell), CD4 (helper T cells), CD8 (killer T cells), Gr-1 (granulocytes) and MAC-1 (macrophage) in normal skin and skin with hyperkeratosis patches at both young and old mice.*

About 30% of 8 month old NF1 epidermal knockout mice developed red, scaly lesions on the back skin, indicating inflammatory responses. It is known that many cancers arise from the sites

of chronic inflammation and infection and many of the inflammation processes are contributing to tumor growth, progression and metastasis (*33*). It is interesting to determine if the loss of NF1 leads to an inflammatory response which contributes to the formation of neurofibroma and other defects in Nf1. We performed immunofluorescence staining on phospho-NFκB, the hub of immune response (*34*), CD3 (T-cell), Gr-1 (granulocytes) and MAC-1 (macrophage) in the skin of newborn, postnatal 7 day, postnatal 21 day and 2.5 month old mice and skin with hyperkeratosis patches of 8 month old NF1 epidermal knockout mice and their wild-type controls. We didn't detect any abnormality till the late stage when the old mice developed skin inflammatory response including an increased level of pNFκB, increased infiltration of immune cells to the dermis as T-cells, granulocytes and macrophages (figure 4A-D). Moreover, mast cells play critical roles in the pathogenesis of Nf1. Neurofibroma composed mainly of abnormal local cells including Schwann cells, endothelial cells, fibroblasts and additionally a large number of infiltrating inflammatory mast cells. Recent works have indicated a role for the microenvironment in plexiform neurofibroma genesis. Emerging evidence pointed to mast cells as crucial contributors to neurofibroma tumorigenesis (*24, 35*). We did toluidine blue staining for mast cells in the skin samples of mice with different age. Only in 8 month old NF1 conditional knockout mice, we observed highly increased mast cell infiltration to the dermis (figure 4E). Our data suggest that NF1 regulates inflammatory response but a second factor such as age contributes to the process.

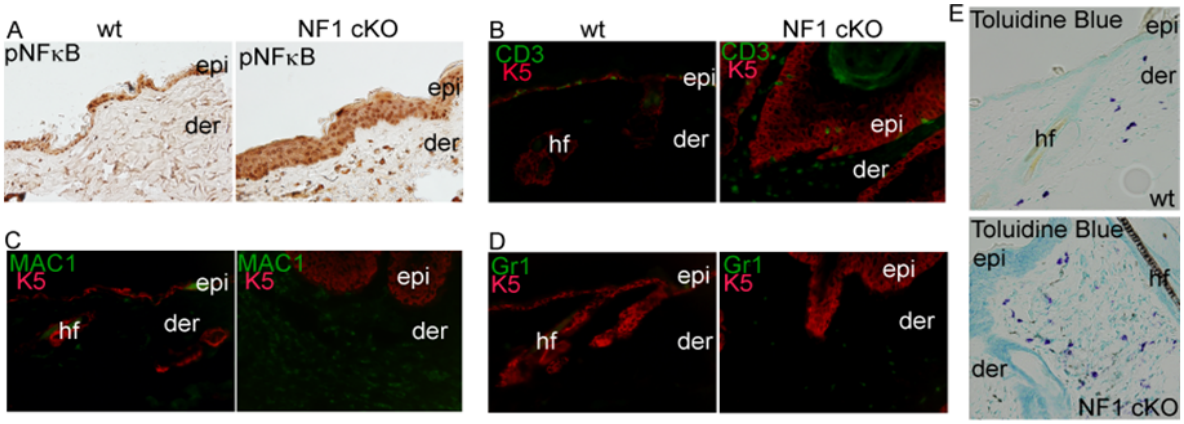

Figure 4: NF1 involves in inflammatory responses. Immunohistochemistry staining of (A) phospho-NFκB, and immunofluorescence staining of (B) CD3 for T-cells, (C) MAC1 for macrophages, (D) Gr-1 for granulocytes (E) Toluidine blue staining (purple cells) for mast cells in an 8 month old NF1 epidermal knockout mouse and a wild-type control. Epi: epidermis, der: dermis, hf: hair follicle.

3d. *Proposed work: Determine the relation between NF1 and psoriasis (months 13-24). We will collect the skin samples from old NF1 epidermal knockout mice and stain with differentiation markers for different layers. We will determine the expression of several psoriasis related genes, such as Th17, IL22, IL23, CD68, CXCL8, CCL2, defensin-2 by quantitative PCR in the skin samples from the lesions in the old mice and compare their expression in the normal skin. We will perform immunofluorescence staining of Th17, IL22 and IL23.*

It is reported that the NF1 expression is downregulated in psoriasis in vivo (*8, 36*, *37*). Psoriasis is a common skin condition that causes skin redness and irritation. Most patients with psoriasis have thick, red skin with flaky, silver-white patches called scales. Psoriasis is a chronic

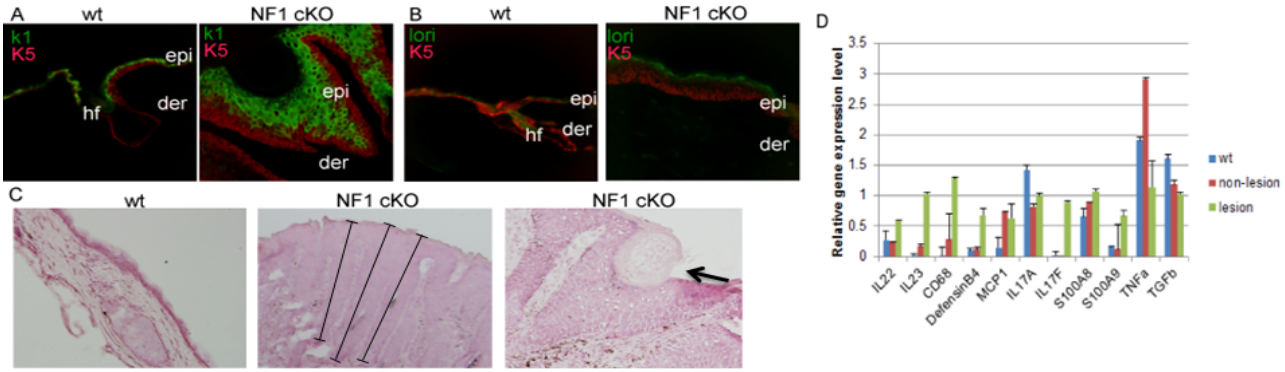

Figure 5: Loss of NF1 induces psoriasis. Immunofluorescence staining of (A) K1 for spinous layer and (B) loricrin for granular layer with K5 for basal layer and H&E staining (C) of an 8 month old NF1 epidermal knockout mouse (NF1 cKO) and a wild-type control. Epi: epidermis, der: dermis, hf: hair follicle. Acanthosis is noticeable in NF1 cKO mice. Elongated 'rete-like' ridges were highlighted and the arrow indicates microabscesses. (D) Quantitative real-time PCR of gene expression involved in psoriasis in 8 month old wild-type (wt) and NF1 epidermal knockout mice (cKO). Gene expression was normalized to transcripts of β-actin.

inflammatory skin disease in which keratinocytes and T cells play major roles in the pathogenesis. As shown in figure 4B, there is an elevated level of T-cell infiltration in old NF1 epidermal knockout mice. One of the hallmarks of psoriasis is the hyper-proliferation of the keratinocytes (*38, 39*). To investigate if old NF1 epidermal knockout mice develop psoriasis, we collected the skin samples from 8 month old mice and stained with k5 for basal layer, k1 for spinous layer and loricrin for granular layer to see which layer(s) are expanded. Not surprisingly, we see increased spinous layer in 8 month old NF1 epidermal knockout mice. Moreover, the basal layer is also expanded but the expression of loricrin is the same compared to wild-type mice (figure 5A, B). Other histological features of human psoriatic skin including acanthosis (epidermal thickening), elongated epidermal rete ridges, hyperkeratosis (cornified layer thickening), parakeratosis (presence of nuclei in cornified layer). And murine psoriasis-like skin presents acanthosis, elongated 'rete-like' ridges, hyperkeratosis, microabscesses of inflammatory cells (neutrophils) and parakeratosis (*40, 41*). From the histological staining by H&E, we saw acanthosis, elongated 'rete-like' ridges and microabscesses (figure 5C). We further examined the expression several genes whose expression is significantly increased in psoriasis skin both in human and mouse, including IL22, IL23, CD68, CCL2, defensing β4 which is the ortholog of human defensing β2, IL17A, IL17F, S100A8, S100A9. We also investigated the expression of TNFα and TGFβ which in general are not highly expressed in psoriasis skin (*42, 43*). In the lesion region of the 8 month old NF1 knockout mice, we detected significant increase of the expression of majority of psoriasis related gene, including IL22, IL23, CD68, CCL2, defensing β4, IL17F, S100A9 but not the control TNFα and TGFβ (figure 5D). The histological staining, immune cells infiltration and the increase expression of psoriasis related gene suggest that loss in NF1 function in epidermis may induce psoriasis. CDSN is an extracellular component of corneodesmosomes which is a critical component for skin barrier formation. CDSN gene is located on chromosome 6, in the major psoriasis susceptibility locus PSORS1. Furthermore, it is known that loss of CDSN leads to psoriasis (*44, 45*). Interestingly, we identified CDSN as a NF1 binding protein by mass spectrometry analysis (table 1). Loss of NF1 in epidermis might result in the malfunction of CDSN which leads to the development of skin psoriasis.

Overall, we identified 22 novel NF1 interacting proteins which involve in Ras signaling pathway, neuron development, metabolism, and skin barrier function, transcription and cytoskeleton association. Although we didn't discover any protein with clear function in differentiation and inflammation, the discovery of the novel NF1 binding proteins might shed lights on

tumorigenesis of neurofibromatosis, cognition defect and loss of NF1 related skin diseases. The analysis of the skin phenotypes of NF1 epidermal knockout mice revealed that loss of NF1 results in increased inflammatory responses in aged mice which are symptoms of psoriasis. Furthermore, NF1 involves in keratinocytes migration and the initiation of differentiation in the epidermis.

In the future, the NF1 interacting proteins will be verified by co-immunoprecipitation and some of the key components will be selected such as CDSN and neuron proteins. The immune-precipitation in this study was done in differentiated mKTs which were established cell lines and didn't represent *in vivo* condition. The mKTs directly from epidermis of wild-type mice should be used for immunoprecipitation study in the future and NF1 epidermal knockout mice mKTs will be used as control. We are confident that promising results would rise from continuous study of this research proposal, and further financial support would be greatly appreciated.

Key Research Accomplishments

- By mass spectrometry analysis of immunoprecipitated NF1 complex, we identified 22 novel NF1 interacting proteins involved in Ras signaling pathway, neuron development, metabolism, and skin barrier function, cytoskeleton association and transcription.

- Through dissecting the role of NF1 in differentiation, we revealed that NF1 involves in keratinocytes migration and the initiation of differentiation in the epidermis.

- We demonstrated that loss of NF1 results in increased inflammatory responses only in old mice suggesting that downregulation of NF1 alone is not sufficient to induce inflammatory response and a second factor such as age contributes to the process.

- We showed that loss of NF1 leads to psoriasis in old mice.

Reportable Outcomes

Developed NF1 epidermal knockout mice and characterized some epidermal phenotypes of the mice specifically in differentiation, inflammation and psoriasis.

Performed immunoprecipitation of NF1 associated complex and mass spectrometry analysis of the proteins. Identified 22 novel NF1 binding proteins.

Conclusion

We tested 6 commercial NF1 antibodies and 22 customized NF1 antibodies and identified a good one for immunoprecipitation.

We did immunoprecipitation of NF1 complex in differentiated mKTs and pulled down some NF1 specific binding proteins. By mass spectrometry analysis, we identified 22 novel NF1 interacting proteins which can be grouped in 7 categories.

After analyzing four different stages of stratification, we suggest NF1 involves in keratinocytes migration and the initiation of differentiation in the epidermis.

Our staining results proved that loss of NF1 results in increased inflammatory responses only in old mice, including an increased level of pNFκB, the hub of immune response and increased infiltration of immune cells to the dermis as T-cells, granulocytes, macrophages and mast cells suggesting that loss of NF1 alone is not sufficient to induce inflammatory response and a second factor such as age contributes to the process.

After analyzing the skin samples from 8 month old NF1 conditional knockout mice, we demonstrated that downregulation NF1 leads to psoriasis in old mice.

References

1. W. Li *et al.*, *Current biology : CB* **15**, 1961 (Nov 8, 2005).
2. B. C. Widemann *et al.*, *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **24**, 507 (Jan 20, 2006).
3. L. C. Krab *et al.*, *JAMA : the journal of the American Medical Association* **300**, 287 (Jul 16, 2008).
4. M. M. Daston *et al.*, *Neuron* **8**, 415 (Mar, 1992).
5. D. H. Gutmann, D. L. Wood, F. S. Collins, *Proc Natl Acad Sci U S A* **88**, 9658 (Nov 1, 1991).
6. L. Alonso, E. Fuchs, *Proc Natl Acad Sci U S A* **100 Suppl 1**, 11830 (Sep 30, 2003).
7. M. Malminen, S. Peltonen, J. Koivunen, J. Peltonen, *BMC Dermatol* **2**, 10 (Aug 29, 2002).
8. J. Koivunen, *Acta Universitatis Ouluensis, Medica* **746**, (2003).
9. J. A. Rothnagel *et al.*, *Differentiation* **65**, 119 (Oct, 1999).
10. K. Takahashi, B. Yan, K. Yamanishi, S. Imamura, P. A. Coulombe, *Genomics* **53**, 170 (Oct 15, 1998).
11. S. Lee *et al.*, *Mol Biol Cell* **16**, 4572 (Oct, 2005).
12. M. P. Washburn, D. Wolters, J. R. Yates, 3rd, *Nat Biotechnol* **19**, 242 (Mar, 2001).
13. T. Ozawa *et al.*, *J Biol Chem* **280**, 39524 (Nov 25, 2005).
14. F. Kweh *et al.*, *Mol Carcinog* **48**, 1005 (Nov, 2009).
15. H. Yla-Outinen *et al.*, *Lab Invest* **82**, 353 (Mar, 2002).
16. B. C. Ling *et al.*, *Cancer Cell* **7**, 65 (Jan, 2005).
17. J. P. Williams *et al.*, *Cell Stem Cell* **3**, 658 (Dec 4, 2008).
18. V. W. Keng *et al.*, *Sarcoma* **2012**, 620834 (2012).
19. E. Fuchs, S. Raghavan, *Nature reviews. Genetics* **3**, 199 (Mar, 2002).
20. T. Scholzen, J. Gerdes, *Journal of cellular physiology* **182**, 311 (Mar, 2000).
21. F. M. Watt, *The EMBO journal* **21**, 3919 (Aug 1, 2002).
22. Y. Huang *et al.*, *Oncogene* **23**, 368 (Jan 15, 2004).
23. T. Ozawa *et al.*, *The Journal of biological chemistry* **280**, 39524 (Nov 25, 2005).
24. F. C. Yang *et al.*, *The Journal of clinical investigation* **112**, 1851 (Dec, 2003).
25. S. Zhang, P. G. Charest, R. A. Firtel, *Curr Biol* **18**, 1587 (Oct 28, 2008).
26. R. Tsuboi, C. Sato, C. M. Shi, H. Ogawa, *J Dermatol* **19**, 652 (Nov, 1992).
27. S. Werner, R. Grose, *Physiol Rev* **83**, 835 (Jul, 2003).
28. L. R. Lund *et al.*, *EMBO J* **25**, 2686 (Jun 21, 2006).
29. G. Zambruno *et al.*, *J Cell Biol* **129**, 853 (May, 1995).
30. H. L. Hsieh, H. H. Wang, W. B. Wu, P. J. Chu, C. M. Yang, *J Neuroinflammation* **7**, 88 (2010).
31. X. Huang, J. Wu, S. Spong, D. Sheppard, *J Cell Sci* **111 (Pt 15)**, 2189 (Aug, 1998).
32. S. Lippens, E. Hoste, P. Vandenabeele, P. Agostinis, W. Declercq, *Apoptosis : an international journal on programmed cell death* **14**, 549 (Apr, 2009).
33. L. M. Coussens, Z. Werb, *Nature* **420**, 860 (Dec 19-26, 2002).
34. W. E. Naugler, M. Karin, *Current opinion in genetics & development* **18**, 19 (Feb, 2008).
35. L. Q. Le, L. F. Parada, *Oncogene* **26**, 4609 (Jul 12, 2007).
36. S. L. Karvonen *et al.*, *The British journal of dermatology* **150**, 211 (Feb, 2004).
37. J. Peltonen, S. L. Karvonen, H. Yla-Outinen, O. Hirvonen, J. Karvonen, *The Journal of investigative dermatology* **105**, 664 (Nov, 1995).
38. M. Kunz, S. M. Ibrahim, *Mediators of inflammation* **2009**, 979258 (2009).
39. B. J. Nickoloff, *Nature medicine* **13**, 242 (Mar, 2007).
40. E. F. Wagner, H. B. Schonthaler, J. Guinea-Viniegra, E. Tschachler, *Nat Rev Rheumatol* **6**, 704 (Dec, 2010).

41. J. E. Gudjonsson, A. Johnston, M. Dyson, H. Valdimarsson, J. T. Elder, *J Invest Dermatol* **127**, 1292 (Jun, 2007).
42. K. Nakajima *et al.*, *J Immunol* **186**, 4481 (Apr 1, 2011).
43. B. J. Nickoloff, *Nat Med* **13**, 242 (Mar, 2007).
44. N. Jonca *et al.*, *Eur J Dermatol* **21 Suppl 2**, 35 (May, 2011).
45. M. Matsumoto *et al.*, *Proc Natl Acad Sci U S A* **105**, 6720 (May 6, 2008).

Appendices

N/A

Supporting Data

Figure 1: Expression of NF1 in murine skin. (A) Murine skin structure. The skin is separated into two compartments, the epidermis and dermis by the basement membrane. The epidermis is stratified epithelia of proliferating and differentiating cells that can be divided into distinct layers by different markers (purple). Adapted from Alonso and Fuchs, 2003 (6). (B) Immuno-fluorescence staining of keratin 6 and NF1 detected by sc67 antibody in skin samples from postnatal 21 day wild-type (wt) and NF1 epidermal knockout mice (NF1 cKO). Epi: epidermis, der: dermis, hf: hair follicle.

Figure 2: Immunoprecipitation of NF1 complex. (A) Increased expression of NF1 after differentiation of mKTs by high calcium and high confluency. (B) Silver staining of immunoprecipitation product after pulldown with NF1 anti-body and rabbit IgG control in differentiated mKTs. Arrows indicate the proteins pull-downed by NF1 antibody only. (C) Western blotting of NF1 in supernatant and immunoprecipitation product after pulldown with NF1 antibody and rabbit IgG control in differentiated mKTs. Arrow indicates NF1 band.

Figure 3: NF1 negatively regulates migration in mKTs. (A) Distance between the explant edge and the most distal cells of the outgrowth was measured over time. (B) Quantitative real-time PCR of gene expression involved in cell migration of newborn epidermis in wild-type (wt), NF1 with heterozygous (het) or homozygous mutation (cKO) mice. Gene expression was normalized to transcripts of β-actin. Gene expression of wt was set at 1.

Figure 4: NF1 involves in inflammatory responses. Immunohistochemistry staining of (A) phospho-NFκB, and immunofluorescence staining of (B) CD3 for T-cells, (C) MAC1 for macrophages, (D) Gr-1 for granulocytes (E) Toluidine blue staining (purple cells) for mast cells in an 8 month old NF1 epidermal knockout mouse and a wild-type control. Epi: epidermis, der: dermis, hf: hair follicle.

Figure 5: Loss of NF1 induces psoriasis. Immunofluorescence staining of (A) K1 for spinous layer and (B) loricrin for granular layer with K5 for basal layer and H&E staining (C) of an 8 month old NF1 epidermal knockout mouse (NF1 cKO) and a wild-type control. Epi: epidermis, der: dermis, hf: hair follicle. Acanthosis is noticeable in NF1 cKO mice. Elongated 'rete-like' ridges were highlighted and the arrow indicates microabscesses. (D) Quantitative real-time PCR of gene expression involved in psoriasis in 8 month old wild-type (wt) and NF1 epidermal knockout mice (cKO). Gene expression was normalized to transcripts of β-actin.

Table 1: Mass spectrometry analysis of the NF1 complex. The NF1 containing complex was immunoprecipitated and analyzed by mass spectrometry to determine the NF1-interacting proteins. Rabbit IgG were used as a control. Proteins that were uniquely identified by anti-NF1 antibody were listed in the table. We divided the proteins to 7 groups some of which were overlapped.